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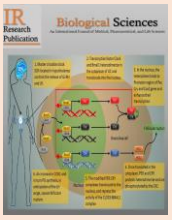

Optimization of growth parameters for enhancing antifungal secondary metabolites of *Talaromyces islandicus* VSGF1 against drug resistant *Candida spp.*

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ABSTRACT

The rapid increase in resistance to antifungal drugs has been raised largely in *Candida* species due to continue and over utilization of available frontline drugs. To overcome this rising health care issue, it's necessary to identify a new and improved antifungal drug without causing any side effects than the existing drugs. In view of this, the present investigation was carried out in findings of the novel antifungal drug against pathogenic Candida species. The fungus *Talaromyces islandicus* VSGF1 was screened for the production of antifungal secondary metabolites through optimization of growth parameters under submerged fermentation by changing one variable at a time. The highest fungal biomass (1.9g/100ml of culture broth) with significant antifungal activity (15 mm zone of inhibition) was achieved in potato dextrose broth supplemented with 2% dextrose at pH6 and 26 OC temperature at stationary condition. A comparative antifungal activity was performed on frontline drugs of amphotericin B, fluconazole, ketoconazole, and nystatin along with extracellular and intracellular crude extracts of *T. islandicus* against eight *Candida* strains. Among the frontline drugs, only ketoconazole exhibited antifungal activity against tested *Candida* spp. and resistance has been observed by amphotericin B, fluconazole, and nystatin. Interestingly, both extracts of *T. islandicus* were found to possess significant and a broad spectrum of inhibitory activity against all tested *Candida* spp. This is the first report on this species from the genus *Talaromyces* that was used for the optimization of process parameters and contribution to the potential producers for antifungal secondary metabolites.

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Introduction

Microbes are evidence for the discovery of pharmaceutical and industrial active natural products. Among the microbial community, fungi are major sources for therapeutic applications for human welfare have been proven from many years. The genus *Aspergillus* and *Penicillium* are the most

Abbreviations: *g/L: Gram's per liter; mm: Milliliter; MTCC: Microbial type culture collection; OC: Temperature; RPM: Rotation per minute; SMF: Submerged fermentation; spp: Species; SSF: Solid state fermentation; ZOI: Zone of inhibition.*

dominant fungi frequently reported for the production of novel pharmacological lead metabolites. Besides that, the maximum number of pharmaceutical active metabolites was also reported from Fusarium, Trichoderma and *Talaromyces* [1]. The recent investigations have been focused on the genus *Talaromyces* is due to production of 316 novel pharmaceutical bioactive products and characteristic pigments [2,3]. A new diphenylketones and xanthone derivatives are isolated from *Talaromyces islandicus* EN-501 exhibited antioxidant and antimicrobial activity, respectively against pathogenic bacteria [4]. Such active biomolecules were reported by more than a single species in the genus *Talaromyces*, but many species have not been screened scientifically and such metabolites are expressed only under unique circumstances. The majority of active metabolites isolated from *Talaromyces* are most effective against bacterial and fungal pathogens [5-10]. Hence, this raised our interest to carry out research on the production of potential antifungal secondary metabolites from the genus *Talaromyces* against *Candida* species.

At present, the invasive *Candida* species are major pathogen causes for superficial to most disseminated bloodstream infections associated with wide clinical manifestations notably in immune-compromised patients. The available antifungal agents of polyenes (amphotericin B, nystatin), azoles (ketoconazole, fluconazole, itraconazole, isavuconazole, posaconazole, and voriconazole) and the echinocandins (anidulafungin, caspofungin, and micafungin) are generally used to treat the fungal infections. However, the majority of these drugs are ineffective in treating immune-compromised patients due to multi drug resistance (MDR) in most of the *Candida* species, particularly in non albican *Candida* species (NAC) such as *Candida glabrata*, *Candida tropicalis*, *Candida auris* and *Candida krusei* [11,12]. A study during 2007-2017 reveals the average isolation rates of *C. albicans* and NAC spp. with 45.8% and 67.3%, respectively. Among NAC spp., fluconazole resistance was higher in C. krusei 97.3% followed by C. glabrata 49.5%, C. tropicalis 34.3% and C. rugosa 33.3% [13]. Therefore, there is an urgent need to undertake the studies on identification of a new antifungal drug, as numerous microbes have proved to produce molecules with a broad spectrum of antimicrobial activity. *Talaromyces* is a mold fungus commonly found in stored cereals. It has a highly versatile metabolism characterized by the secretion of numerous biopolymers degrading enzymes, mycotoxins and anthraquinones that altogether offers a wide spectrum of potential industrial applications [14].

In view of this, the present research was carried out on novel antifungal producing secondary metabolites from *Talaromyces islandicus* VSGF1 against pathogenic *Candida* species.

Materials and Methods

Talaromyces islandicus VSGF1

The fungus T. islandicus VSGF1 (VSGF1; Prescribed laboratory code for fungal isolate) was previously isolated from vermicompost soil of Kalaburagi region, Karnataka-India and screened for the production of antifungal secondary metabolites. The molecular identification confirmed its identity as *Talaromyces islandicus* through the internal transcribed spacer of the conserved ribosomal DNA. The sequence was submitted to Gen bank accession number MN818685.1 (**Figure 1a, b,** & **c**).

Test organisms

Eight *Candida* strains from four species namely *Candida* albicans MTCC:1966, C. albicans MTCC:2795, C. albicans MTCC:3019, C. glabrata MTCC:3814, C. glabrata MTCC:3981, C. tropicalis MTCC:1406, C. tropicalis MTCC:230 and C. haemulonii MTCC:8303 were used as the test organisms. The clinical isolates were obtained from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Medical Technology (IMTECH), Chandigarh, India.

Chemicals

Agar, Czapek dox agar, Dextrose, Dipotassium phosphate, Ethyl acetate, Ferrous sulphate, Fructose, Glucose, Magnesium, Magnesium sulfate, Maltose, Martin Rose Bengal, Oat meal, Peptone, Potassium chloride, Potassium phosphate, Sodium nitrate, Starch agar, Sucroce, Yeast extract were purchased from Himedia Laboratory Pvt. Ltd. Amphotericin B, Chloramphenicol, Fluconazole, Ketoconazole, and Nystatin were supplied by Sigma Aldrich.

Optimization of Culture Media

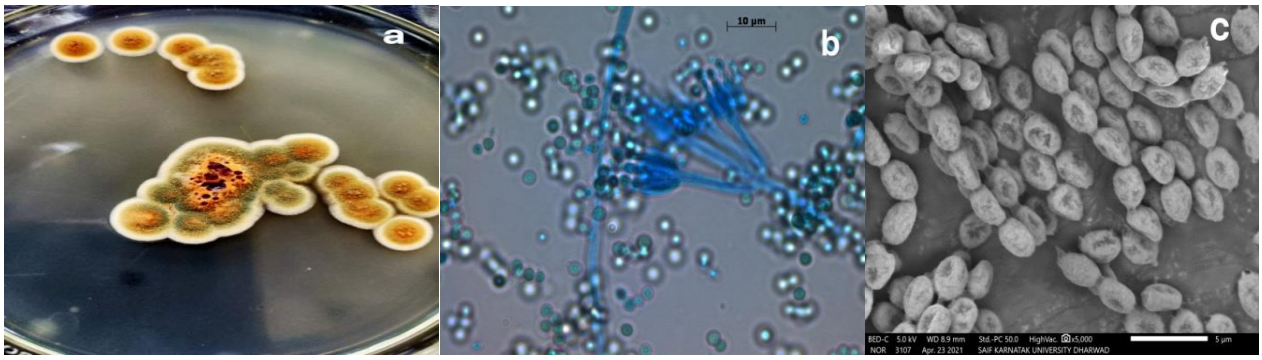

Figure 1. a) Culture plate of *T. islandicus* VSGF1 on potato dextrose agar medium showing colony color, growth pattern and sporulation; inserted image showing reverse plate with pigmentation b) Fungal mycelium with phialides containing conidiophores producing conidia c) chains of single celled conidia

Initially, the culture medium was optimized by using eight different media (Gram's/Liter of distilled water) namely potato dextrose agar (PDA; potato-200g, dextrose-20g, agar-15g), czapek dox agar (CZA; sucroce-30g, sodium nitrate-2g, dipotassium phosphate-1g, magnesium 0.5g, potassium chloride-0.5g, ferrous sulphate-0.01g, agar-15g), malt extract agar (MEA; malt extract-30g, peptone-5, agar-15), potato carrot agar (PCA; potato-200g, carrot-250g, agar-15g), sabouraud dextrose agar (SDA; dextrose-40g, peptone-10, agar-15g), starch agar (SA; 30g), oatmeal agar (OA; oat meal-30g, agar-15g) and Martin Rose Bengal agar media (MRBA; peptone-5, dextrose-10, potassium phosphate-1g, magnesium sulfate-0.50g, rose Bengal-0.05, chloramphenicol-0.10g, agar-15) were used to determine the good growth and sporulation of *T. islandicus* VSGF1 [15]. The freshly sub cultured fungus was inoculated in each petriplate containing different medium was incubated at 28 OC and regular observations were made in 7-10 days. The medium which supports the better growth and sporulation were further subjected to broth cultures for extraction of secondary metabolites to screen the antifungal activity against each one of the *Candida* species.

Optimization of Growth Parameters

The optimization of culture medium was performed using conventional methods by changing various parameters such as carbon, nitrogen, pH, temperature, incubation period and agitation for the production of highest biomass and antifungal secondary metabolites.

Effect of Carbon Sources

Various carbon sources such as dextrose, fructose, glucose, maltose (simple carbon) and sucrose (complex carbon) were used for the optimization. The Erlenmeyer flask containing 100ml of basal medium added with each carbon source (2 % weight/volume) were inoculated with 5 mm diameter of fungal disc and incubated at 28 OC for 7days [16]. The biomass was harvested through centrifugation, washed two to three times with deionized water and removed the excess water through blotting filter paper. Then, the biomass was weighed as g/100 ml of broth culture. The filtered broth was treated with an equal volume of ethyl acetate (organic solvent) and the solvent phase was collected and condensed to form a crude extract. The obtained crude extract was subjected to antifungal activity against each one of the *Candida* spp.

Effect of Nitrogen Sources

The each 0.5% (w/v) of peptone and yeast extract was served as nitrogen sources were added to the basal medium with 2% of dextrose as carbon source and medium without nitrogen source used as a control [15]. The flasks were inoculated with 5 mm fungal mycelium and incubated at 28 OC. After 7 days, the amount of biomass and antifungal activity was recorded as mentioned above.

Effect of pH

The five conical flasks containing 100ml of basal medium was adjusted to various pH from 4-8 by adding 0.1N NaOH or 0.1N HCl. The 5mm diameter of the fungal mycelium disc was inoculated and incubated at 26 OC [17]. After 7days, the effect of pH was determined through the weight of biomass production and highest antimicrobial activity.

Effect of Temperature

The fungus was subjected to different temperature ranges from 25 to 30 OC to examine the optimum temperature required for the growth and yield of antifungal metabolites. 100ml of basal medium amended with 2% dextrose was inoculated with 5mm diameter of fungal mycelium and incubated at provided temperature [18]. After 7 days of

incubation, the weight of fungal mycelium and zone of inhibition was recorded.

Effect of Incubation Period

To study the optimum incubation time, the fungus was inoculated in basal medium and incubated at 26 OC with a different time period of 5, 7, 9, 11, 13 & 15 days. The fungal growth and production of secondary metabolites were kept under continuous observation [19]. After the incubation period, the amount of biomass and zone of inhibition was assessed.

Effect of Agitation

Five flasks containing 100ml of basal medium supplemented with 2% dextrose was inoculated with 5 mm disc of fungal mycelium and incubated at 26 OC for 7 days at the agitation speed of 100, 120, 140, 160, 180 and 200 rotation per minute (rpm) on a rotary shaker [19]. The flask without agitation kept as control. The weight of biomass and the effect of agitation on antimicrobial activity were determined.

Preparation of Fungal Crude Extracts and Standard Drugs

Talaromyces islandicus was subculture on freshly prepared PDA medium. From this, 5 mm diameter of mature fungal colony was inoculated in 1000 ml Erlenmeyer flask containing 500 ml of optimized PDB with pH6 and incubated at 26 OC for 10 days. The fungal biomass was separated by centrifugation at 15,000 rpm for 10 min. The obtained supernatant was subjected to liquid-liquid extraction with an equal amount of ethyl acetate in separating funnel. The organic layer was separated, then condensed to form crude and used as extracellular extract (Ex). The separated fungal mycelium was washed repeatedly to remove media components and dried at 60-80 OC in hot air oven. Then, the dried mycelium was crushed in pistil and mortar in order to extract intracellular secondary metabolites by adding ethyl acetate and centrifuged. The supernatant obtained was collected and evaporated to dryness. The obtained crude was used as intracellular extract (In). Approximately, the amount of crude extract from extracellular and intracellular solvent extraction yielded 0.7-0.8 g/L and 0.25-0.30 g/L, respectively. The both fungal extract and antifungal drugs of amphotericin B, ketoconazole, fluconazole and nystatin were dissolved in mg/ml of dimethyle sulfoxide (DMSO) as a test samples [20].

A Comparative Antifungal Activity of Fungal Crude Extracts with Standard antifungal Drugs

The antifungal activity was carried out against eight *Candida* strains of C. albicans 1966, C. albicans 2795, C. albicans 3019, C. glabrata 3814, C. glabrata 3981, C. tropicalis 1406, C. tropicalis 230 and C. haemulonii 8303 by using agar well diffusion assay [21,22]. Sterilized yeast extract peptone dextrose agar medium (YPDA; Yeast extract-10g/l, peptone-20g/l, dextrose-20g/l, agar-15g/l of distilled water, pH 6.8) was used to perform the antifungal activity. 24 h old freshly prepared *Candida* cultures were seeded on petriplate containing YPDA medium. Seven wells of each 5 mm in diameters were made with sterile cork borer. Two wells were loaded with each 50µl of extracellular (Ex) and intracellular (In) test samples of T. *islandicus* and other wells were loaded with standard drugs of amphotericin B, ketoconazole, fluconazole and nystatin, whereas DMSO was used as negative control and incubated at 28 OC for 24 h. The efficacy of test samples and standard drugs was measured by zone of inhibition (ZOI) using Antibiotic Zone Scale-C PW297 (Himedia Laboratory Pvt. Ltd).

Statistical analysis

The biomass yield was recorded by drying the fungal mycelium at 60-80 OC and obtained weight was expressed as g/100ml of culture broth. The antifungal activity of standard antifungal drugs and extract of T. islanicus were assessed by means ± SD (Standard deviation); using Microsoft Excel.

Results and Discussion

Optimization of Growth Parameters

The microbial optimization process is required to check the effect of various nutrients and physical environmental factors on production of novel metabolites having various biological activities are mainly based on particular strain to be used. During optimization, the both solid state fermentation (SSF) and submerged fermentation (SMF) methods were employed for extraction of secondary metabolites. The solid-state fermentation (SSF) was not much supports for the secondary metabolite production, whereas highest metabolite yield was observed in submerged fermentation (SMF). Noura et al., successfully employed the production of β-glucosidase through solid state fermentation by species *Talaromyces* pinophilus, thus the fermentation process is largely depends on the type of strain to be used [23]. The basic production of fungal bioactive products has largely been produced by liquid based submerged fermentation have been reported (SMF) [24]. However, conventional technology for the production of valuable fungal products is mainly obtained in liquid

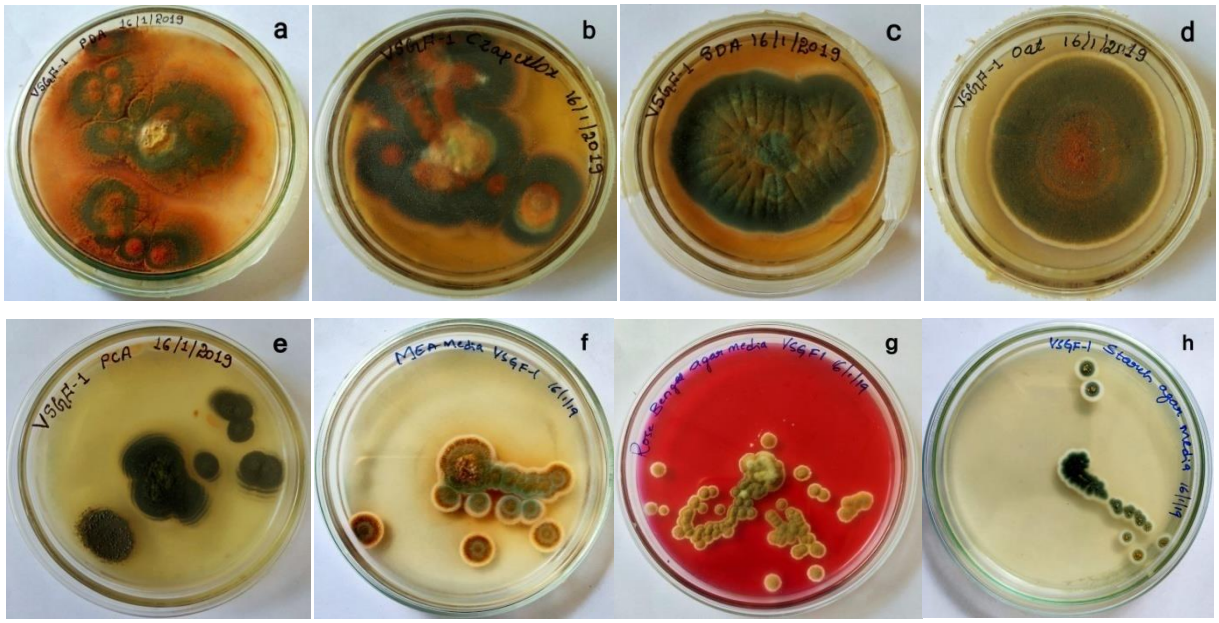

Figure 2. The mycelium growth and sporulation of T. islandicus VSGF1 on different nutrient media after 7 days of incubation period was observed on a) Potato dextrose agar b) Czapek dox agar c) Sabouraud dextrose agar d) Oat meal e) Potato carrot agar f) Malt extract agar g) Martin rose Bengal agar and h) Starch agar.

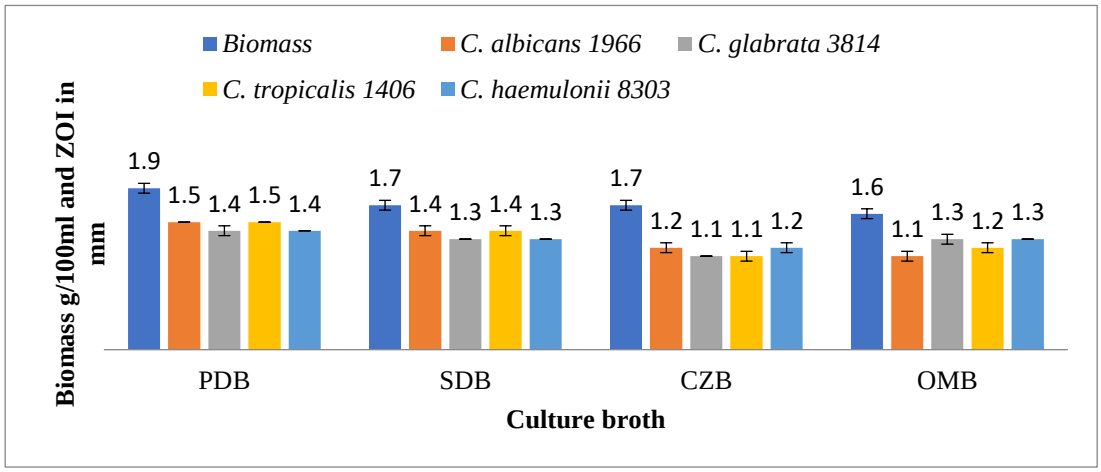

Figure 3. Effect of different broth cultures on production of fungal biomass and antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

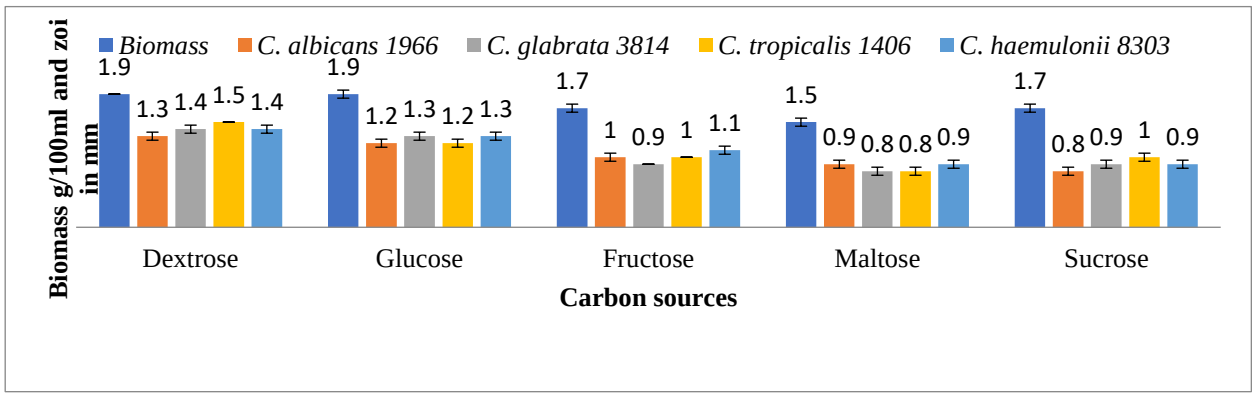

Figure 4. Effect of simple and complex carbon sources were studied on production of fungal biomass and antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

submerged fermentation [25]. Hence, the SMF method was employed for the extraction of crude extract throughout the studies.

Optimization of Culture Media

Initially, the culture medium was optimized for the maximum production of biomass and highest yield of antifungal secondary metabolites against human pathogenic *Candida* spp. Among the eight media used, potato dextrose agar medium was found suitable for the fast and better growth of *T. islandicus* with characteristic soluble pigment as compared to other media. Whereas, czapekdox medium, sabouraud dextrose medium and oatmeal medium also enhanced the better growth of mycelium then next to PDA medium (**Figure 2a, b, c** & **d**). Whereas, the poor and slow growth was observed in potato carrot agar, malt extract agar, Martin rose Bengal agar and starch agar (Fig. 2e, f, g & h). Mikio et al., reported that, T. bacillosporus was grown on medium amended with malt extract supports the pigment extraction along with antibacterial activity [26]. In present studies, the malt extract containing medium enhances the pigment production with reduced amount of biomass as well as antifungal activity. The effect of PDA medium on fungal growth revealed a strong exponential growth up to 7 days and reached to stationary phase on 9th day of incubation period. The highest secondary metabolite secretion was observed in medium supplemented with dextrose and maximum biomass was obtained in media containing sucrose along with supplementary substances like dipotassium phosphate, ferrous sulfate, magnesium, potassium chloride and oatmeal. Similarly, among the broth used, potato dextrose broth (PDB) produced highest 1.9 g of biomass yield and followed by sabouraud dextrose broth (SDB) 1.7 g, czapek dox broth (CZB) 1.7g and Oat meal (OMB) 1.6g/100ml of culture broth. As compared to antifungal activity, the highest 15 mm ZOI was recorded by PDB and followed by SDB 14 mm against C. albicans 1966 and C. tropicalis 1406. The OMB and CZB extract showed 13 mm and 12 mm against C. glabrata 3814 and C. haemulonii 8303, respectively (**Figure 3**). The variation observed in fungus growth and activity among the broth used is due to utilization of nutrients or may be related to media composition in which the fungus was grown. The similar studies were reported with maximum mycelium weight and inhibitory bioactive metabolites in potato dextrose broth against Klebsilla oxytoca, Staphylococcus aureus and Bacillus subtilis, respectively [15,27]. The maximum biomass and highest activity of *T. islandicus* VSGF1 was achieved significantly in potato dextrose medium, hence the medium was further used to optimize other cultural parameters for better growth and production of antifungal secondary metabolites.

Effect of Carbon Sources

Among the carbon sources used, the highest 1.9g/100ml of fungal biomass and significant antifungal activity was achieved in basal medium amended with 2% dextrose followed by glucose. During the optimization studies, both simple and complex carbon sources were examined and found highest of 15±0 mm ZOI against C. tropicalis 1406 in media supplemented with dextrose (**Figure 4**). The low activity was recorded in medium supplemented with maltose. The glucose at 2% concentration also yielded 1.9g/100ml of biomass and showed reduced ZOI of 13±0.057 mm against C. glabrata 3814 and C. haemulonii 8303 as compared to dextrose. In the present investigation, the 2% carbon concentration was optimum for the growth of fungal biomass and antifungal activity was also found maximum at similar concentration. Carbon sources are major nutritive components and building blocks of overall growth and development of microbes and each carbon sources play a different role thus enhances the production of novel secondary metabolites. Asnaashari et al., who has reported the highest production of antibiotic penicillin was achieved in medium supplemented with lactose as a carbon source [16]. Verma et al., reported that, the endophytic species of Aspergillus isolated from Calotropis procera showed the highest antimicrobial metabolites in culture medium supplemented with starch [28].

Effect of nitrogen Sources

In the present optimization process, the nitrogen sources have found a negative impact on production of fungal biomass along with antifungal activity. Among the nitrogen sources used, quite better activity was recorded from yeast extract as compared to peptone. The medium supplemented with yeast extract showed the reduced amount of biomass 1.2g/100ml and 9±0.057 mm ZOI against C. haemulonii 8303 (**Figure 5**). As the concentration of nitrogen sources increased, the fungal biomass decreased. In the present study, only carbon source alone supported for the production of biomass as well as antifungal metabolites. It has been also reported that some nitrogen sources have inhibitory action on production of some secondary metabolites and also antimicrobial agents due to sugar catabolic repression and acid accumulation resulting in oxygen depletion and pH imbalance. The optimization studies reported on Aspergillus sp. CRP5 showed 3g/L of yeast extract influences the secondary metabolite production [28]. The utilization of nitrogen source is generally depends on the type of microorganism, most of the microbes

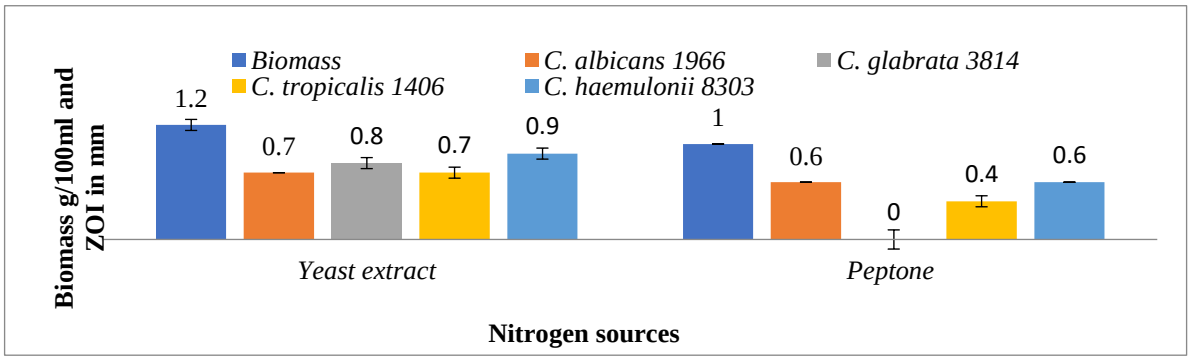

Figure 5. Effect of various nitrogen sources were investigated on production of fungal biomass and antifungal activity against pathogenic Candida spp. [Data expressed as mean ± SD (n=3)].

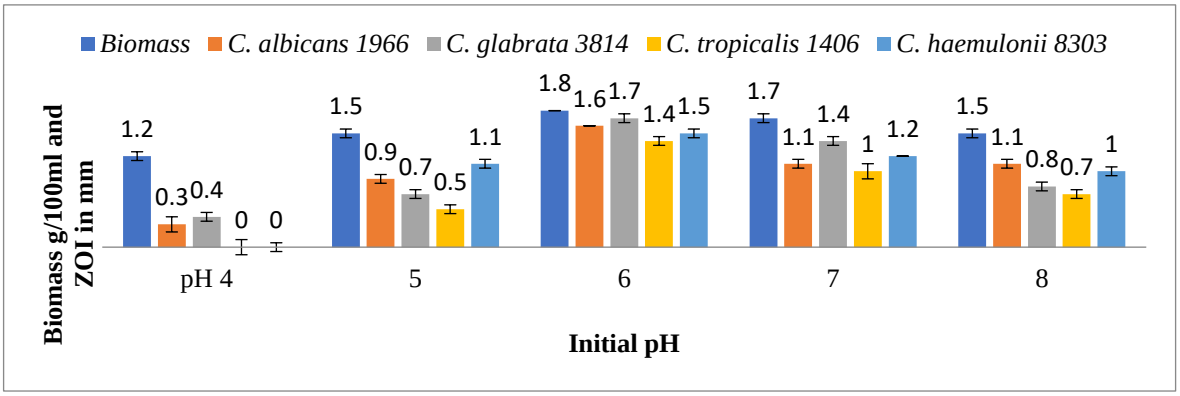

Figure 6. Effect of different pH was determined for maximum production of fungal biomass and highest antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

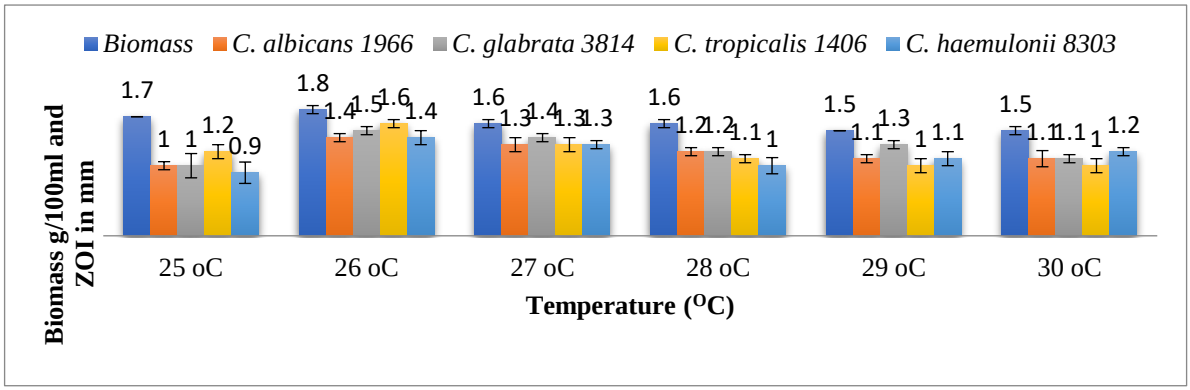

Figure 7. Effect of temperature ranges from 25 to 30 OC was studied on production of fungal biomass and antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

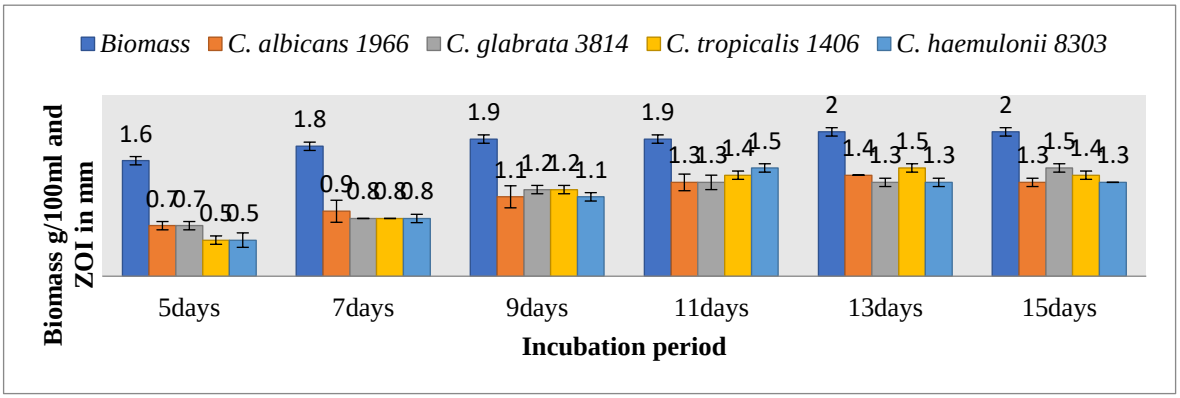

Figure 8. Effect of incubation period from 5 to 15 days at difference of one was recorded on production of fungal biomass and antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

metabolized the nitrogen sources to produce various cell components [29]. The remarkable characteristic feature of fungi is their extraordinary flexibility with respect to carbon and nitrogen sources. The requirement of carbon and nitrogen (C:N) ratio varied among the fungal species, thus the growth characteristics might be strain dependent [30].

Effect of pH

The pH of medium plays a vital role in the overall growth and development of micro-organisms. The T. islandiucs was grown at varying pH from 4 to 8 at difference of one and found maximum 1.8g/100ml of biomass production along with, the highest of 17±0.057 mm and 16±0 mm ZOI formed against C. glabrata 3814 and C. albicans 1966, respectively at pH 6 (**Figure 6**). In case of pH4 the poor growth was observed, whereas at pH 5 and 8 the biomass production was very less and produced a small rounded white colony about 3-5 mm in diameter. The pH 6 of the basal medium was found to be maximum growth with higher antifungal activity and near to neutral pH 7 also influenced the biomass growth and support the antifungal activity after pH6. A slight variation in pH of the medium significantly reduces the metabolite production and affects the cellular process such as biosynthesis and regulation of secondary metabolites [31]. Hence, the pH is most important in determining cultural parameter due to permeability characteristic of the cell wall and cell membranes are relatively depends on cell environment pH level. Similar studies were reported by Gogoi et al., on Fussarium solani produced maximum secondary metabolites at pH 6 and other studies also supports the present investigation [32,33,34]. Most of the fungal strain has its specific pH in which it grows and reproduces. Generally, fungi prefer an acidic pH for good growth and activity. Usually too alkaline and too acidic solutions showed a negative impact on fungal growth and pH from 4 to 9 is favorable for the growth of fungi [35,36] and change in pH of the medium induces the production of new metabolite may lead to adverse effect on antibiotic production.

Effect of temperature

T. islandicus was grown at temperature ranges between 25 to 30 OC at a difference of one to the basal medium. The maximum fungal biomass 1.8g/100ml and significant antifungal activity of 16±0.057 mm was found against C. tropicalis 1406 at 26 OC (**Figure 7**). Whereas, at temperature 25 OC also supports the maximum production of biomass with less antifungal activity and vice-versa 27 OC showed highest activity with less biomass production. Thus, the ttemperature has profound effect not only in the production of metabolites as well as in the morphological and physiological aspects of the micro-organisms [37]. Similar studies were reported by Ritchie et al., and Jain et al., produced maximum growth and highest antimicrobial activity at temperature 25 OC during the optimization of Rhizoctonia solani and Aspergillus terreus [18,33]. In most optimization studies, the temperature 26 OC was optimum for the production of bioactive secondary metabolites and supports the good growth of fungal biomass [28]. Compaore et al., reported that the highest production of antimicrobial substances by Aspergillus fumigates was recorded at temperature 37 OC [19]. When temperature below 20 OC, less biomass production and low activity was reported and above 40 OC fungal growths was stopped, thus the low temperature may reduce the metabolic activity and high temperature kills the fungal cells [32].

Effect of incubation period

The production of maximum 2g/100ml of biomass was recorded on 13th and 15th day of incubation period, whereas the highest antifungal activity of 15±0.057 mm ZOI was recorded from 9th, 13th and 15th day of incubation period against C. heamulonii 8303, C. tropicalis 1406 and C. albicans 3019, respectively (**Figure 8**). As the incubation period increases the production of biomass increased, whereas the antifungal activity remains the same as the 9 to 15th day of incubation. Hence, the suitable incubation time for the production of highest biomass and antimicrobial secondary metabolites was observed at 9th day. The incubation period directly affects the total growth and overall biological process of the fungi and the fungus which was incubated for 9-10 days produced maximum secondary metabolites in provided culture medium have been reported [28]. The fungal biomass and production of secondary metabolites remain constant after 10 days of incubation period.

Effect of agitation

The *T. islandicus* was subjected to different agitation speed and showed maximum 1.8g/100ml of biomass production with 14±0.057 mm ZOI against C. haemulonii 8303 at 160 rpm (Fig. 9). However, the highest of 16±0.057 mm ZOI against C. tropicalis 1406 along with 1.9 g/100ml of fungal biomass production was observed in culture broth kept under stationary condition. Agitation helps in equal distribution of nutrients and aeration to increase the rate of metabolites in the fermentation medium and it regulates the oxygenation rate in an Erlenmeyer flask. The high agitation may damage the fragmented hyphae and affects the cellular metabolism, thus consequently reduce the antibiotic production [19]. In the present study, the

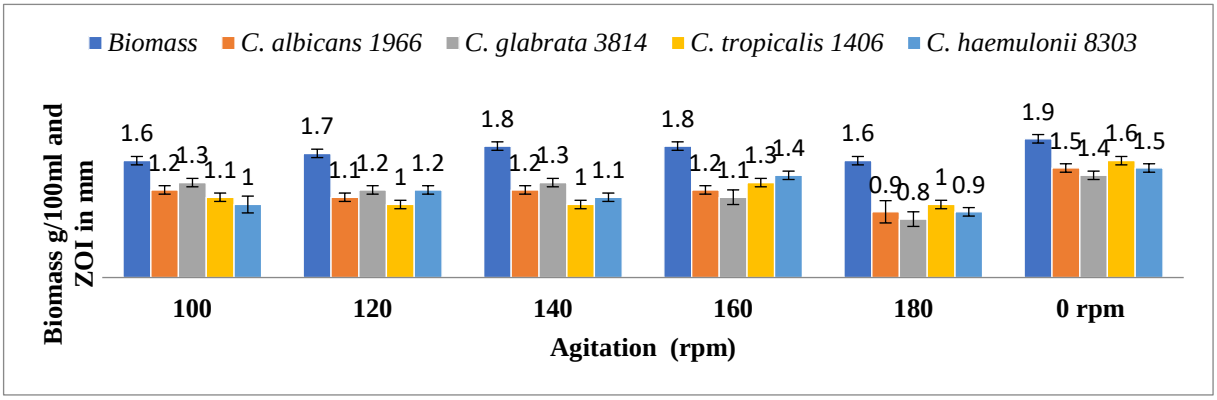

Figure 9. Effect of different agitation speed along with control static condition was assessed on production of fungal biomass and antifungal activity against Candida spp. [Data expressed as mean ± SD (n=3)].

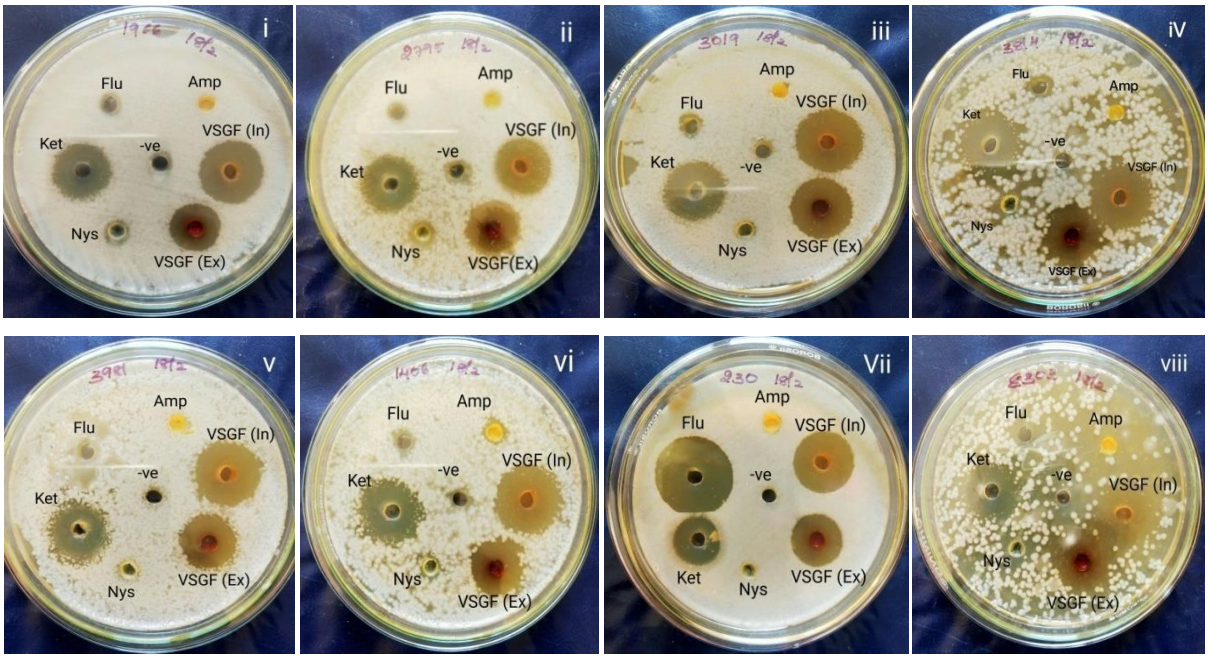

Figure 10. Antifungal activity of T. islandicus VSGF1 [VSGF (In) - intracellular extract of T. islandicus VSGF1; VSGF (Ex) - extracellular extract of T. islandicus VSGF1; -ve-negative control (DMSO)] with standard antifungal drugs [Amp-Amphatericin B, Flu-Fluconazole, Ket-Ketoconazole, Nys-Nystatin] showing zone of inhibition against tested Candida spp. i) C. albicans MTCC1966, ii) C. albicans MTCC2795 iii) C. glabrata MTCC3019 iv) C. glabrata MTCC3814 v) C. glabrata MTCC3981 vi) C. tropicalis MTCC1406 vii) C. tropicalis MTCC230 viii) C. haemulonii MTCC8303

Table 1. A Comparative antifungal activity of *Talaromyces islandicus* VSGF1 with standard antifungal drugs

***Candida spp.* MTCC**	**Amp-B**	**Flu**	**Ket**	**Nys**	***T. islandicus* VSGF (In)**	***T. islandicus* VSGF (Ex)**
C. albicans 1966	---	---	14.33±0.57	---	16.66±0.57	13.33±0.57
C. albicans 2795	---	---	14	---	15.33±0.57	13.33±0.57
C. albicans 3019	---	---	14.33±0.57	---	16.33±0.57	15.66±0.57
C. glabrata 3814	---	---	13.33±0.57	---	17.66±0.57	13.66±0.57
C. glabrata 3981	---	---	13.33±0.57	---	16.33±0.57	13.33±0.57
C. tropicalis 1406	---	---	13.33±0.57	---	15	13.33±0.57
C. tropicalis 230	---	19.33±0.57	12.33±0.57	---	15	14.33±0.57
C. haemulonii 8303	---	---	13.33±0.57	---	15.33±0.57	13.33±0.57

*Note: Amp-B: Amphatericin B, Flu- Fluconazole, Ket-Ketoconazole, Nys-Nystatin, Ex-Extracellular, In-Intracellular [n=3; mean ± SD].

maximum amount of biomass and antimicrobial substances were produced at stationary condition.

The current studies on optimization of growth parameters influenced the maximum production of fungal biomass as well as the highest antifungal secondary metabolites. Initially, when the *T. islandicus* was grown in potato dextrose broth at pH 6.8 and temperature 28 OC produced maximum 1.7/100ml of biomass and highest of 14 mm ZOI against tested *Candida* spp. Whereas, after the optimization of PDB, the production of fungal biomass increased 1.9 to 2g/100ml and antifungal activity from 14 mm to 17 mm, thus showed the promising results against drug resistant *Candida* spp., as compare to frontline antifungal drugs. Hence, in the present investigation the pH and temperature played a major role in enhancement of the highest secondary metabolite production in the culture broth.

A Comparative Antifungal Activity of Fungal Crude Extracts with Standard Drugs

The antifungal activity of extracellular and intracellular crude extract of *T. islandicus* VSGF1 showed promising results by exhibiting highest activity when compared to standard drugs of amphotericine B, fluconazole, ketoconazole and nystatin. The ethyl acetate crude intracellular extract showed highest of 17.66±0.57 mm ZOI against C. tropicalis 1406 and followed by 16.66±0.57 mm against C. albicans 1966, C. albicans 3019 and C. glabrata 3981. Whereas, the extracellular extract exhibited maximum of 15.66±0.57 mm ZOI higher than that of the standard ketoconazole 14.66±0.57 mm against, C. albicans 1966, C. albicans 3019, C. tropicalis 1406 and C. tropicalis 230 (**Table 1** & **Figure 10**). The intracellular extract of *T. islandicus* showed highest antifungal activity whereas extracellular extract exhibited equal activity when compare to ketoconazole.

Among the standard drugs, only ketoconazole was found susceptible to all tested *Candida* species with significant inhibitory activity. However, fluconazole exhibited susceptibility only against the single test organism C. tropicalis 230 with 19.33±0.57 mm ZOI. The results revealed the development of resistance to tested *Candida* species at drug concentrations of mg/ml of amphotericine B, fluconazole and nystatin. Interestingly, at the same concentration of extracellular and intracellular crude extract of *T islandicus* VSGF1 showed significant inhibitory activity against all tested *Candida* spp. All the antifungal agents tested in the present study have a documented clinical effect on *Candida* spp. In present study, the *Candida* species exhibited resistant to amphotericin-B, fluconazole and nystatin is may be due to continue screening of clinical isolates to the antifungal drugs may sometimes lead to development of resistance among the *Candida*. Overuse of azole drugs has an important role in the development of fluconazole resistance in *Candida* spp. This resistance has an increasing trend among C. albicans and non-albicans *Candida* species over recent decades [11]. However, C. glabrata is highly resistant to fluconazole, with a global resistance rate of 15.7% as compared to other *Candida* species. Primary resistance to fluconazole is rare in C. albicans (1.4%) and C. tropicalis (4.1%) [38]. Nolte et al., reported the isolation of AmB-resistant in C. albicans from patients with leukemia [39]. The lack of activity of AmB is described in systemic murine fusarial infection as well as in clinical isolates [40,41]. Powderly et al., noted that there is a correlation between failure of AmB therapy and in vitro resistance in yeasts isolated from cancer patients with invasive fungal infection [42]. Azoles (fluconazole and ketoconazole) and polyenes (amphotericin-B and nystatin) are the two most frontline antifungal drugs in hospitals and increasing resistance to both frontline antifungals severely hampers our ability to treat fungal disease. Combination therapy could be a promising method to increase the effectiveness of antimicrobial agents. The combination of two or more antifungal drugs or non-antifungal drugs has been appraised towards to overcome the therapeutic failures [43,44]. This necessitates the search for novel bioactive metabolite with potential antimicrobial agent. The fungus *T. islandicus* extracts alone had a stronger effect against tested *Candida* isolates than its combination with common antifungal drugs and may lead to discovery of a strong and effective antifungal agent. However, this finding needs future studies to be further clarified.

The genus *Talaromyces* is well known for the production of effective antibacterial and antifungal secondary metabolites have been previously reported and thus support the present investigation [45,46,47]. The Talaroconvolutins and Talaroderxines obtained from T. convolutes and T. derxii exhibited inhibitory activity against *Candida* albicans and Bacillus subtilis, respectively [48,49]. The T. flavus strains have been reported to be effective against Verticillium, Aspergillus fumigates and C. albicans [50,51,52]. Hence, the current research was carried out on *T. islandicus* VSGF1 to screen against pathogenic *Candida* spp. and it has been confirmed that the *T. islandicus* is a potential soil fungus for the production of antifungal secondary metabolites against the broad spectrum of *Candida* spp.

Conclusion

The present investigation was successful in producing the highest biomass with the maximum

antifungal activity of *T. islandicus* through optimization of growth parameters. The fungal crude extracts have shown promising antifungal activity against drug resistance *Candida* spp. and confirmed the growth inhibition in C. albicans, C. glabrata, C. tropicalis and C. haemulonii, who have developed multidrug resistance against frontline drugs of amphatericin B, fluconazole and nystatin. The results will serve as a base to identify the lead biomolecules useful in the production of new antifungal drugs to deal the infections caused by drug resistant *Candida* species. The bioactive metabolites produced by T. islandicus VSGF1 proved to exhibit potent antifungal effect against pathogenic *Candida* species; hence these secondary metabolites would be used for the treatment of infections caused by above mentioned *Candida* species, after identifying its chemical nature and scientific validation through clinical studies.

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Conflicts of interests

Nil

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